Volume 260, number 1, 19–22 FEB 07992 January 1990

The protonated form of 1-N^6-etheno-[erythro-9-(2-hydroxy-3-nonyl)] adenine is identified at the active site of adenosine deaminase

Valeria R. Caiolfa*, David Gill and Abraham H. Parola*

*Departments of *Chemistry and Physics, Ben-Gurion University of the Negev, Beer-Sheva 84 105, POBox 653, Israel*

Received 9 August 1989; revised version received 21 September 1989

A novel fluorescent competitive inhibitor of adenosine deaminase (EC 3.5.4.4) (ADA), 1-N^6-etheno-[erythro-9-(2-hydroxy-3-nonyl)] adenine (ϵ-EHNA), is protonated at the active site of the enzyme. In ϵ-EHNA [$K_I = (4.06 \pm 1.00)\ 10^{-6}$ M] part of the competive inhibition of EHNA is combined with spectroscopic properties of etheno-adenines. Computer subtraction of the fluorescence excitation spectrum of ADA from that of its equimolar complex with ϵ-EHNA yielded the corrected excitation spectrum of ϵ-EHNA *at the active site* of the enzyme. This spectrum mimics that of ϵ-EHNA at pH 5.5 in buffer solution and is suggested to indicate a shift in protonation equilibrium at the active site.

Adenosine deaminase; 1-N^6-Etheno-[erythro-9-(2-hydroxy-3-nonyl)] adenine; Etheno-adenine; Fluorescent inhibitor; pH indicator; Fluorescence polarization

1. INTRODUCTION

Adenosine deaminase (EC 3.5.4.4) (ADA) is important for its metabolic role [1–6] (it is absent in severe combined immunodeficiency [7]), quaternary polymorphism [8,9], response to malignancy [3,5] and availability of tight inhibitors [10]. Still unknown are the amino acid composition at the active site and the regulatory role of polymorphism.

EHNA, a classic example of rational design of enzyme inhibitors by Schaeffer et al. [11] is a tight competitive inhibitor of ADA ($K_I = 1.6.10^{-9}$ M) [12], shown to bind to a hydrophobic region adjacent to the catalytic site [13].

Fluorescent etheno-adenosine derivatives, initially introduced by N. Leonard [14], have been used to probe adenine-containing systems, be it nucleic acids or energy conserving substrates. Etheno-adenosine, obtained by reaction of aqueous chloroacetaldehyde with adenosine, is a modification of adenosine at the 6-NH_2 group. It is a highly fluorescent compound with a quantum yield close to 0.6 and a lifetime of 23 ns [15].

We report here a novel fluorescent competitive inhibitor of (ADA) [1], 1-N^6-etheno-[erythro-9-(2-hydroxy-3-nonyl)] adenine (ϵ-EHNA), which combines properties of the two aforementioned groups. Moreover, it indicates protonation at the active site of ADA.

Ground state pH indicator equilibria are usually detected by absorbance (color). When one is compelled to use fluorescent indicators for the sake of sensitivity, the corrected fluorescence excitation spectra are identical with the absorbance and can yield the same information. This rule, however, is not obeyed in etheno adenines [14], which also have other spectroscopic peculiarities [16–18]. Nevertheless, the fluorescence excitation spectra are pH dependent and can be calibrated with reference to buffered solutions. Thus, the corrected fluorescence excitation spectrum of ϵ-EHNA bound to ADA at the active site resembles that of ϵ-EHNA in buffer solution at pH 5.5. This spectrum is observed although the pH of the buffered bulk is 7.5.

The pH dependence of the corrected fluorescence excitation spectra of ϵ-EHNA suggests the following ground state protonation equilibrium (fig.1; [19]).

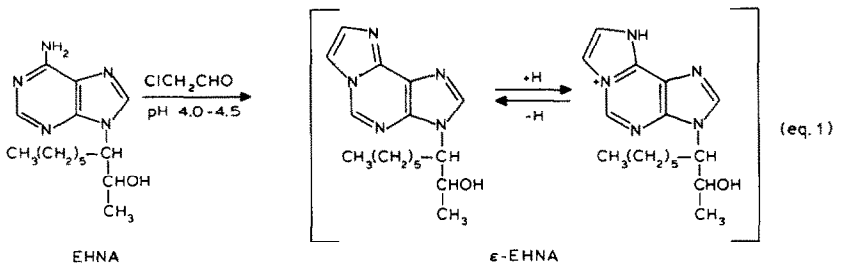
2. MATERIALS AND METHODS

EHNA was prepared according to Shaeffer and Schwender [11] and Giner-Sorolla [20], with a few modifications [21]; ϵ-EHNA was obtained by dissolving EHNA (100 mg) in freshly prepared chloroacetaldehyde (1.57–1.00 M, pH 4.5) according to Keeler and Campbell [22]. Chloroacetaldehyde in aqueous solution at pH 4.5 was made according to Secrist iii et al. [14]. The mixture was stirred 48 h at room temperature in the dark at constant pH = 4.0–4.5 and checked by TLC and U.V. absorbance. Purification was by preparative TLC on cellulose. The fluorescent band was extracted with distilled water, and cellulose was separated by centrifugation (9980 × g). ϵ-EHNA was recrystalized from ethanol and diethyl ether. The single fluorescent spot on TLC (eluent CH_2Cl_2/CH_3OH, 90/10, v/v) had $R_f = 0.500$ [10]. ^{1}H NMR (DMSO-d6) ppm 9.44 (s, 1H), 8.53 (s, 1H),

Correspondence address: A.H. Parola, Department of Chemistry, Ben-Gurion University of the Negev, Beer-Sheva 84 105, PO Box 653, Israel

Abbreviations: ADA, adenosine deaminase; ϵ-EHNA, 1-N^6-etheno-[erythro-9-(2-hydroxy-3-nonyl)] adenine; EHNA, erythro-9-(2-hydroxy-3-nonyl)] adenine; 2′-deoxycoformycin (Pentostatin), [3-(2′-deoxy-β-D-erythro-pentofuranosyl)-3,6,7,8-tetrahydroimidazol[4,5-d][1,3]diazepin-8-(R)-ol]. The abbreviation 'ϵ' was introduced by Secrist et al. [14] and stands for etheno.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50



8.25 (d, 1H), 7.21 (d, 1H), 4.38 (m, 1H), 4.00 (m, 1H), 3.5 (s, OH), 0.78-1.80 (m, 16H).

Adenosine deaminase (high purity grade, from calf intestine, approximately 200 units/mg protein, Sigma [8]) activity was determined by spectrophotometric assay at 265 nm [9] and by the Berthelot colorimetric assay for ammonia [8]. Enzyme kinetics were run at 24 ± 1°C. The concentration of ADA was calculated assuming $M_r = 45\,000$. ADA was incubated 5 min with either ε-EHNA, EHNA or ε-adenosine prior to addition of adenosine. In the spectrophotometric method the concentration of adenosine was varied from $1.4 \cdot 10^{-5}$ to $1.6 \cdot 10^{-4}$ M.

Photobleaching of ADA was carried out according to the procedure described by Parola et al., (Anal. Biochem., in press).

Corrected fluorescence excitation spectra were recorded on an SLM 4800 spectrofluorometer with a depolarizer at the exit of the excitation monochromator, at an emission wavelength of 400 nm. A concentrated solution of Rhodamine B in propylene glycol was used as reference.

Fluorescence lifetimes were measured on the SLM4800 phase-modulation spectrofluorometer as described before [26]. At least 5 measurements of phase and modulation were accumulated at 6, 18 and 30 MHz in accumulation time windows of 25 s, and converted into τ_{phase} and τ_{mod} by SLM lifetim softwar on an Apple IIe computer. Heterogeneity analysis of τ_{phase} and τ_{mod} data was carried out by least-squares analysis program ISSC6-87, assuming phase and modulation frequency independent measurement errors, $\sigma_{phase} = 0.2$ and $\sigma_{modulation} = 0.004$, respectively [23]. Fluorescence decay curves were obtained in a nanosecond single photon correlation spectrofluorometer. The sample was excited with the 316 nm nitrogen emission line [24].

Steady-state fluorescence polarization measurements of ε-EHNA as a function of ADA concentration, at 22 ± 1°C, was measured by: (i) Perkin-Elmer MPF-44 equipped with dichroic polarizers and emission depolarizer, at $\lambda_{ex} = 315$ nm, $\lambda_{em} = 400$ nm, excitation and emission slits were 6 and 20 nm respectively; (2) SLM 4800, T-configuration, equipped with dichroic polarizers and depolarizer at the exit of the excitation monochromator, at $\lambda_{ex} = 315$ nm, with either 350 nm or 400 nm Kodak Wratten sharp cut-off filters in front of the emission photomultipliers. Samples were prepared from freshly bleached ADA in 63 mM sodium phosphate buffer, pH 7.5. The concentration of ε-EHNA was kept constant in all samples ($5 \cdot 10^{-6}$ M in phosphate buffer pH 7.5) while ADA concentration was varied from $3.33 \cdot 10^{-7}$ M to $9.45 \cdot 10^{-6}$ M. The concentration of ADA was determined from the blanks (equal concentration of enzyme in the absence of ε-EHNA) by the spectrophotometric activity assay, assuming a molecular weight of 45 000.

Dynamic polarization of ε-EHNA as a function of ADA/ε-EHNA molar ratio was measured on SLM 4800 at $\lambda_{ex} = 315$ nm, at modulation frequencies of 6 and 30 MHz and T-configuration (in accumulation time windows of 25 s, with a depolarizer at the exit of the excitation monochromator and Kodak Wratten sharp 400 nm cut-off filter in front of the emission photomultipliers). Measurements were recorded either by rotating the excitation polarizer from 0° to 90° with polarizer in channel A at 0° and polarizer in channel B at 90° or, by rotating channel B polarizer from 0° to 90° with polarizers in channel A and excitation both at 0.

3. RESULTS

The complicated spectroscopic properties of ε-EHNA are similar to those of other ε-adenine analogs [14,15]. U.V. absorption spectra are pH-dependent and show an isosbestic point at 292–294 nm (fig.1), indicating equilibrium between protonated and non-protonated forms (eq.1). The absorptivity of the strongest band, $\epsilon_{276\ nm} = 3570\ M^{-1}cm^{-1}$, is inconveniently low, half that of ε-adenosine [14]. Ground state acidity constant, $pK_a^{\epsilon\text{-EHNA}} = 4.03 \pm 0.19$, was derived from absorbance vs pH at four different wavelengths. Upon excitation at 315 nm, vibrationally unresolved fluorescence emission with $\lambda_{max} = 407$ nm was obtained. Fluorescence lifetime measured by single photon correlation and phase modulation fluorometry [25] yielded a single lifetime $\tau = 27.1 \pm 0.2$ ns, at pH 7.5. Fluorescence intensity was also pH dependent and a plot of peak height vs pH yielded $pK_a^{\epsilon\text{-EHNA}} = 4.2$, close to that in absorption (they do not have to be equal). Fig.1 shows corrected fluorescence excitation spectra as a function of pH. A

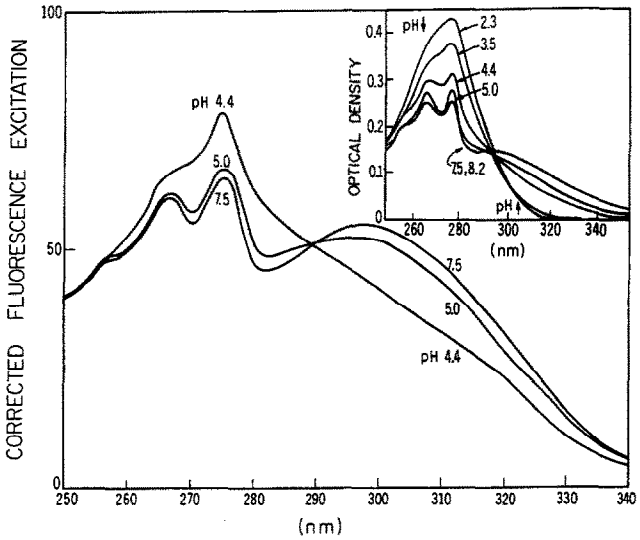


Fig.1. Corrected fluorescence excitation spectra of ε-EHNA as a function of pH, fluorescence emission at 400 nm. Insert: Dependence of U.V. absorbance of εEHNA on pH in buffer. 22 ± 1°C; [ε-EHNA] = 0.1 mM. Left of isosbestic point: protonated species; right: unprotonated. Fluorescence excitation differs from UV absorption in that the unprotonated form (e.g. pH 7.5) contributes more to fluorescence than the protonated one.

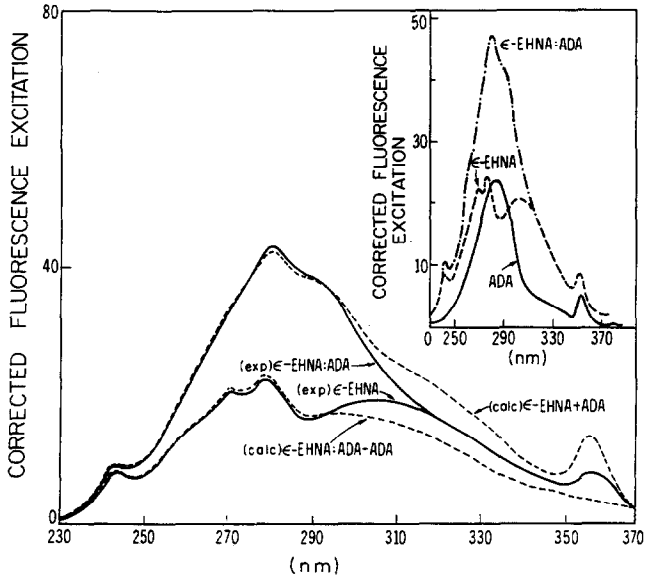


Fig.2. (Insert) Corrected fluorescence excitation spectra, in phosphate buffer at pH 7.5, at $\lambda_{em} = 400$ nm and at room temperature, of: (- · -) ϵ-EHNA:ADA 1:1 complex, 1 μM; (---) ϵ-EHNA, 1 μM; (——) ADA, 1 μM ($M_r = 45\,000$ [24]; laser photobleached, see section 2 and [26]. (Figure) Full lines: expanded view of ϵ-EHNA:ADA 1:1 complex and ϵ-EHNA of insert. Lower dashed curve: computer subtraction of fluorescence excitation spectrum of ADA from that of the 1:1 complex between ϵ-EHNA and ADA of insert. This curve (denoted in the figure as (calc)ϵ-EHNA:ADA-ADA) is the spectrum of ϵ-EHNA at the active site of ADA. The abscence of the Raman line at 360 nm validates the subtraction procedure. This curve mimics that of ϵ-EHNA in buffer at pH 5.5. Upper dashed curve: computer addition (from insert) of ϵ-EHNA, in buffer at pH 7.5 and ADA, in buffer at pH 7.5. This reference spectrum (denoted in the figure as (calc)ϵ-EHNA + ADA) represents a non-interacting 1:1 mixture. All spectra were free of inner filter effect (absorbance < 0.2).

linear Perrin plot of ϵ-EHNA in glycerol (50% in phosphate buffer) yielded limiting anisotropy value $r_0 = 0.23$.

ϵ-EHNA is a competitive inhibitor of ADA, $K_I = (4.0 \pm 1.0) \cdot 10^{-6}$ M. Binding of ϵ-EHNA to photobleached ADA (to eliminate non-peptidic fluorescent impurity in ADA; the enzyme is unharmed [26]), was studied by steady-state and phase modulated fluorescence polarization. We observed binding saturation beginning at 0.5 ADA/ϵ-EHNA molar ratio. While no change in fluorescence emission maxima was noted, the following lifetimes were observed: $\tau_1 = 24.60 \pm 1.06$ ns and $\tau_2 = 2.90 \pm 0.42$ ns with fractional contributions of 0.80 and 0.20 ($\pm$ 0.02) respectively. Binding specificity to the active site of ADA was confirmed by competition experiments in the presence of 2′-deoxycoformycin[1] ($K_I = 2.5 \cdot 10^{-12}$ M [12]), EHNA ($K_I = 1.6 \cdot 10^{-9}$ M [12] and adenosine; complete loss of fluorescence polarization resulted from competition of inhibitors with ϵ-EHNA, while a $\times 30$ concentration of adenosine ($K_m = 2.5 \times 10^{-5}$ M) was required for 90% reduction in polarization.

Fig.2 shows corrected fluorescence excitation spectra of ϵ-EHNA bound in 1:1 molar ratio to ADA. The decline in fluorescence at $\lambda = 300$ nm relative to that at 276 nm indicates a shift in the protonation equilibrium at the active site of ADA, which resembles that observed in buffered solution at pH = 5.2–5.5 (fig.1).

4. DISCUSSION

EHNA, the precursor of ϵ-EHNA, had been shown to have a peculiar mechanism of inhibition [27]. The initial step, in which the hydrophobic chain and its hydroxyl group are thought to be involved, obeys the classical mechanism of competitive inhibition and is characterized by a K_I of $2 \cdot 10^{-7}$ M. Then a consecutive rearrangement of either the enzyme or the inhibitor occurs, yielding a tight ADA-inhibitor complex. Thus EHNA is a strong inhibitor of ADA (overall inhibition constant $1.61 \cdot 10^{-9}$ M) in which, remarkably, the target 6-NH_2 is unaffected.

Evidently, the conformational modifications occurring in the ADA-EHNA encounter complex must be different from those [28] which, in the ADA-adenosine complex, yield the transition-state structures which progress towards the product. The distortions of the native configurations might determine the destination of a molecule as an inhibitor or a substrate.

The initial characteristics of binding of EHNA to ADA may be retained in ϵ-EHNA, in which the hydrophobic moiety has not been altered. Thus, the inhibitor senses the environment of the enzyme active site, although the protonation to which it is subjected may not be directly related to the catalytic mechanism of ADA.

Our results may support the theoretical prediction of Warshel [29], who suggested that enzymes can be viewed as 'super solvents'. The protein can stabilize[2] ionic states more effectively than aqueous solutions do. Having identified the protonation of ϵ-EHNA, one cannot state where the proton comes from. Thus, the lowering of pH at the active site would imply the presence of a local pool rich in H_3O^+ segregated from the bulk of the buffer. Alternatively, direct protonation by amino acid residues may be involved. Whichever is the proton donating moiety, fluorescence excitation of ϵ-EHNA, measured at steady-state conditions, probes an equilibrium and not transients related to catalysis. In this equilibrium, the ratio of non-protonated to protonated ϵ-EHNA is 25:1, while at the buffered bulk pH of 7.5 this ration is ~3100:1. Structural restrictions and polar interactions could also, in principle, shift the fluorescence spectra. Yet, the fluorescence spectra of ϵ-adenine analogues are known to be particularly insen-

[1] 2′-Deoxycoformycin (Pentostatin) is the strongest known inhibitor of ADA. It is a transition state inhibitor with unique affinity to the active site.

[2] or destabilize the neutral form. In either case $\Delta pK_a \approx 2$ is observed, corresponding to 3 kcal/mole.

sitive to solvent polarity [14,16]. The only documented environmental influences on the fluorescence spectra are pH and, to a much lesser extent, solvent viscosity [14].

Etheno-adenine analogues have found wide use in cAMP binding, release and exchange studies [15]. Fluorescent ϵ-adenylated enzymes (i.e. glutamine synthetase) were used for the determination of distances between metal ion binding sites and fluorescent nucleoside. Etheno-adenosine analogues have also been used for the determination of binding regions of the corresponding nucleosides (ATP, AMP) in adenylate kinase. These studies were carried out by means of fluorescence quenching, energy transfer of U.V. difference spectroscopy.

To the best of our knowledge, this is the first report where the peculiar proton sensitivity of an etheno analogue has been applied to probe the 'acidity' of an enzyme active site by means of the relatively sensitive steady-state fluorescence excitation spectroscopy.

The fluorescence excitation spectrum of enzyme bound ϵ-adenosine (the etheno modification of natural substrate, adenosine) could not be obtained. Although ϵ-adenosine has an intact ribose moiety, it shows lower affinity to ADA ($K_I = 3 \cdot 10^{-5}$ M)[3]. Therefore, the requirement of equilibrium conditions[4] between ϵ-adenosine and ADA could not be fulfilled within a concentration range free of inner filter effect[5]. Thus, as far as ADA is concerned, the choice of ϵ-EHNA was advantageous.

Acknowledgements: We are indebted to Mr Max Enkin for having written the computer program and to Professors S. Bitner, D. Kost and J. Becker for their advice and help. This work was supported in part by grant N00014-89-J-1625 of the Office of Naval Research.

REFERENCES

[1] Berne, R.M., Rall, T.W. and Rubio, R. (1983) in: Regulatory Function of Adenosine, Nijhoff, The Hague.
[2] Martin, D.W. jr. and Gelfand, E.W. (1981) Annu. Rev. Biochem. 50, 845–877.
[3] Smith, S.D., Shatsky, M., Cohen, P.S., Warnke, R., Link, M.P. and Glader, B.E. (1984) Cancer Res. 12 5657.
[4] Babusikova, O., Hrivnakova, A., Pechan, I. and Kand Ujhazy, P. (1984) Neoplasma 31, 661.
[5] Balis, M.E. (1985) Ann. NY Acad. Sci. 451, 142–149.
[6] Schrader, W.P., Stacy, A.R. and Pollara, B. (1976) J. Biol. Chem. 251, 4026–4032.
[7] Martin, D.W. jr. and Gelfand, E.W. (1981) Annu. Rev. Biochem. 50, 845–886.
[8] Weisman, M., Caiolfa, V.R. and Parola, A.H. (1988) J. Biol. Chem. 263, 5266–5270.
[9] Porat, N., Gill, D. and Parola, A.H. (1988) J. Biol. Chem. 263, 14608–14611.
[10] Plunkett, W. and Cohen, S.S. (1975) Cancer Res. 35, 1547–1554.
[11] Schaeffer, H.J. and Schwender, C.F.J. (1974) J. Med. Chem. 17, 6–8.
[12] Agarwal, R.P. and Parks, R.E. (1978) J. Methods Enzymol. 51, 502–507.
[13] Schaeffer, H.J. and Vogel, D. (1965) J. Med. Chem. 8, 507.
[14] Secrist iii, J.A., Barrio, J.R., Leonard, N.J. and Weber, G. (1972) Biochemistry 11, 3499–3506.
[15] Leonard, N.J. (1984) Crit. Rev. Biochem. 15, 125–199.
[16] Spencer, R.D., Weber, G., Tolman, G.L., Barrio, J.R. and Leonard, N.J. (1974) Eur. J. Biochem. 45, 425–429.
[17] Inoue, Y. and Kuramochi, T. (1979) Biopolymers 18, 2175–2194.
[18] Vanderkooi, J.M., Weiss, C.J. and Woodrow iii, G.V. (1979) Biophys. J. 25, 263–275.
[19] Scheller, K.H. and Sigel, H. (1983) J. Am. Chem. Soc. 105, 3005–3014.
[20] Giner-Sorolla, A. (1980) European Patent Application 79193233.7.
[21] Caiolfa, V.R. (1989) Ph. D. Thesis, Ben-Gurion University.
[22] Keeler, E.K. and Campbell, P. (1974) Biochem. Biophys. Res. Commun 72, 575–580.
[23] Lakowicz, J.R., Laczko, G., Cherek, H., Gratton, E. and Limkeman, M. (1984) Biophys. J. 46, 463–477.
[24] Skorka, G., Shuker, P., Gill, D., Zabicky, J. and Parola, A.H. (1981) Biochemistry 20, 3103–3109.
[25] Spencer, R.D. and Weber, G. (1969) Ann. NY Acad. Sci. 158, 361–376.
[26] Parola, A.H., Caiolfa, V.R., Bar, I. and Rosenvaks, S. (1989) Anal. Biochem., in press.
[27] Frieden, C., Kurz, L.C. and Gilbert, H.R. (1980) Biochemistry 19, 5303–5309.
[28] Wolfeden, R., Wentworth, D.F. and Mitchell, G.N. (1977) Biochemistry 16, 5071–5077.
[29] Warshel, A. (1981) Biochemistry 20, 3167–3177.
[30] Pesce, A.J., Rosen, C.G. and Pasby, T.L. (1981) in: Fluorescence Spectroscopy, Dekker, New York.
[31] Schroeder, F. (1988) in: Methods for Studying Membrane Fluidity, pp. 193–217, Liss, New York.

[3]We have studied the binding of ϵ-adenosine by Michaelis-Menten kinetics and found that it also is a competitive inhibitor of ADA.
[4]The equilibrium binding conditions are achieved when the initial concentration of the enzyme is of the same order of magnitude as the dissociation constant of the ligand–enzyme complex, which is in this case $\sim 10^{-5}$ M [30].
[5]The absorbance of the solution in the given range of wavelengths must be less than 0.2 [31].